Available online at www.sciencedirect.com

ScienceDirect

Biochimica et Biophysica Acta 1783 (2008) 263–274

Biochimica et Biophysica Acta BBA

www.elsevier.com/locate/bbamcr

Tip30-induced apoptosis requires translocation of Bax and involves mitochondrial release of cytochrome *c* and Smac/DIABLO in hepatocellular carcinoma cells

Mei Shi *, Shi-Gan Yan, Shu-Tao Xie, Hong-Na Wang

School of Life Science, Shandong University, 27 ShanDa South Road, Jinan 250100, Shandong, China

Received 12 June 2007; received in revised form 5 October 2007; accepted 10 October 2007
Available online 24 October 2007

Abstract

TIP30 (Tat-interacting protein 30), a newly found proapoptotic factor, appears to be involved in multiple functions including metabolic suppression, apoptosis induction, and diminishing angiogenic properties. In the present study, we reported that mitochondrial events were required for apoptosis induced by TIP30 in hepatocellular carcinoma cells (HCC cells). Translocation of Bax was essential for TIP30-induced apoptosis, whereas overexpression of the anti-apoptotic protein Bcl-xL delayed both second mitochondria-derived activator of caspases (Smac/DIABLO) release and onset of apoptosis. Furthermore, TIP30-induced apoptosis was dependent on caspase activity because the pan-caspase inhibitor benzyloxycarbonyl-Val-Ala-Asp (OMe)-fluoromethyl ketone (z-VAD-fmk) blocked DNA fragmentation. Release of Smac/DIABLO from the mitochondria through the TIP30–P53–Bax cascade was required to remove the inhibitory effect of XIAP (X-linked Inhibitor of Apoptosis) and allowed apoptosis to proceed. Our results showed for the first time that Bax-dependent release of Smac/DIABLO, cytochrome *c* and AIF from the mitochondria mediated the contribution of the mitochondrial pathway to TIP30-mediated apoptosis. Our data suggested that adenovirus-mediated overexpression of TIP30 was capable of inducing therapeutic programmed cell death *in vitro* by activating the mitochondrial pathway of apoptosis. On the basis of these studies, elucidating the mechanism by which TIP30 induces cell death might establish it as an anticancer approach.

Keywords: Hepatocellular carcinoma; TIP30; Bax; Bcl-xL; Cytochrome *c*; Smac/DIABLO

1. Introduction

TIP30 (also known as CC3), 242-amino-acid long, is evolutionary conserved and expressed ubiquitously in human tissues and some tumor tissues with serine/threonine kinase properties. CC3 was first identified as a metastasis suppressor for small cell lung carcinoma (SCLC) and predisposed cells to apoptosis in response to death signals. Subsequently, CC3 was independently identified as a protein named TIP30, which enhanced human immunodeficiency virus-1 (HIV-1) Tat-activated transcription by phosphorylating the heptapeptide repeats of the C-terminal domain (CTD) of the largest RNA polymerase II subunit [1,2]. It was a putative metastasis suppressor that promoted apoptosis and inhibited angiogenesis [3,4]. TIP30-deficient mice had a high susceptibility of hepatocellular carcinoma and other tumors. Therefore, some carcinomas harbored missense mutations in the *tip30* gene [5–7]. Furthermore, studies showed that TIP30 was an important proapoptotic factor that accounted for significant growth retardation in small cell lung carcinoma (SCLC), which could be largely mediated by the ability of TIP30 to promote apoptosis [2,8]. Moreover, ectopic expression of TIP30 in SCLC cells induced a number of apoptosis-related genes, such as *Bad* and *SIVA* [9]. Nevertheless, the molecular mechanism by which the TIP30-mediated apoptosis had remained largely unknown.

Recently, induction of TIP30 in tumor cells was shown to correlate with chemosensitivity to 5-FU. These data implicated exogenous expression of TIP30 sensitized HCC cells to cytotoxic drugs and to apoptosis induced by tumor necrosis factor-related ligands *in vitro* [10]. Later studies showed that TIP30 might abolish its native tumor-suppressor activity and gain oncogenic activities partially through up-regulation of N-cadherin, thereby

* Corresponding author. Tel.: +86 531 88364326; fax: +86 531 88564326.
E-mail address: clubstone@sdu.edu.cn (M. Shi).

0167-4889/$ - see front matter © 2007 Elsevier B.V. All rights reserved.
doi:10.1016/j.bbamcr.2007.10.006

potentiating the pathogenesis of HCC in patients [11]. Our studies recently showed that *tip30* gene transferred by adenovirus-mediated effectively induced apoptosis in HCC cells in a time-dependent manner. TIP30 predisposed HCC cells to apoptosis according to the P53 pathway. The tumor-suppressor P53 could induce growth arrest and apoptosis in response to a number of cellular stresses. The results showed that when HepG2 cells were infected with Ad-TIP30, levels of wide P53 were increased in a time-dependent manner with an asynchronous apoptosis. We further analyzed *p53* mRNA level by real-time PCR. The *p53* mRNA was significantly enhanced after Ad-TIP30 infection. The *Bax* gene promoter was highly P53-responsive and its expression was up-regulated by P53. We further evaluated the role of Bax in Ad-TIP30-mediated apoptosis. Total Bax levels were elevated 2-fold higher than controls, demonstrating that the influence upon Bax was caused by asynchronous activation of P53. Consistent with the elevation of Bax, a decrease of Bcl-xL was found in cells infected by Ad-TIP30 [10,12]. Although the functions of many molecules and genetic pathways involved in TIP30-mediated apoptosis have begun to be established, our understanding of the details remains fragmented.

Apoptosis is induced in response to a variety of environmental stressors such as heat shock, radiation, various chemotherapeutic agents, and oxidative stress [13,14]. The two major apoptotic pathways involve either mitochondria or death receptors. In the mitochondria, death signals lead to changes in mitochondrial membrane permeability and the subsequent release of proapoptotic factors, including cytochrome *c*, apoptosis inducing factor (AIF), second mitochondria-derived activator of caspase (Smac/DIABLO), and endonuclease G. This leads to the cytoplasmic assembly of procaspase-9, cytochrome *c*, and apoptosis protease-activating factor 1 (Apaf-1) into an initiation complex known as the apoptosome [15–17]. Formation of the apoptosome leads to the activation of caspase-9 and subsequent activation of "executioner" caspases such as caspase-3, which is blocked by the inhibitor-of-apoptosis proteins (IAPs). The IAP family of proteins regulates apoptosis by preventing the action of the central execution phase of apoptosis through direct inhibition of the effector caspase-3 and/or caspase-7. In addition, they prevent initiation of the intrinsic caspase activation cascade by directly inhibiting both apical and effector caspases [18,19]. Smac/DIABLO functions to promote caspase activation by inhibiting IAP family proteins, thereby relieving the block on caspase activation [20,21]. Moreover, AIF and endonuclease G translocate directly to the nucleus where they induce chromatin condensation and/or DNA fragmentation [22].

Mitochondria play a pivotal role in regulating apoptosis. The key regulatory proteins of mitochondria-mediated apoptosis are the Bcl-2 family of proteins, which can either promote cell survival (Bcl-2 and Bcl-xL), or induce cell death (Bax and Bak). Bcl-2 and Bcl-xL are required for the maintenance of mitochondrial integrity by inhibiting the mitochondrial release of proapoptotic factors. On the other hand, Bax and Bak are sufficient to initiate the loss of outer mitochondrial integrity, resulting in apoptosis [23–25]. Bax is distributed in many tissues and promotes apoptosis in a wide variety of cell types. Upon signal stimulation, Bax translocates to mitochondria where it facilitates the release of cytochrome *c*. More recently, studies have provided direct evidence that Bax is required for the execution of the intrinsic apoptotic pathway in response to certain anticancer agents [26,27]. Bcl-xL can be detected in numerous tumor cell lines, particularly in HCC cells. In contrast, it exerts an anti-apoptotic effect by blocking Bax translocation to the mitochondria, preserving mitochondrial integrity and preventing the subsequent release of apoptogenic molecules [28,29].

To date, a substantial literature has detailed many specific biochemical events that occurred upon TIP30 in a few cell types showing apoptotic characteristics. In most cases, these reports dealt with a relatively limited portion of a clearly multiple step process. Accordingly, how these individual events are coupled to more proximal and distal ones is not fully understood. Our previous studies supported that P53 played a vital role in TIP30-mediated proapoptotic activity. In this study, we construct replication-defective adenoviral vectors containing the *tip30* gene or *lacZ* gene. To further study the TIP30-mediated apoptotic pathway, we analyze the translocation of Bax, release of Smac/DIABLO and elimination of XIAP to caspases in HCC cells. In the current study, we demonstrate the status of mitochondria and its downstream effectors in TIP30-mediated pathway. In particular, the data help to detail a sequence of events that proceeds from the translocation of Bax through the release of cytochrome *c* to activation of caspases.

2. Materials and methods

2.1. Antibodies and reagents

Antibodies against cytochrome *c* were purchased from Oncogene Research Products, (San Diego, CA); caspase-3, -8, -9, poly (ADP-ribose) polymerase, Smac/DIABLO, XIAP, and AIF were all purchased from Sigma (St Louis, MO). All antibodies were diluted 1:2000 or 1:1000, in BSA (diluted in TBST). Secondary antibodies were diluted 1:000 or 1:2000 with 5% non-fat milk (diluted in TBST). Z-LEHD-fluoromethyl ketone (z-LEHD-fmk) and benzyloxycarbonyl-Val-Ala-Asp (OMe)-fluoromethyl ketone (z-VAD-fmk) were also purchased from Sigma (St Louis, MO).

2.2. Recombinant adenoviruses and infection protocol

The recombinant adenovirus vectors expressing human TIP30 cDNA were constructed by standard protocols as described previously [31,32]. The resultant viruses were named Ad-TIP30. An adenovirus vector carrying *LacZ* gene was used for monitoring infection efficiency. All vectors were propagated in 293 cells, purified, and stored at −80 °C, as described previously [31].

2.3. Cell culture conditions and establishment of HepG2 cells stably expressing Bcl-xL

HCC cells: HepG2 and HepG2 cells transfected with control vector or Bcl-xL were maintained in six-well plates with 2 ml of Dulbecco's Modified Eagle's Medium (DMEM) containing 10% fetal bovine serum (FBS) under an atmosphere of 5% CO_2. Medium of transfected cells was supplemented with 1 mg/ml G418 (Life Technologies, Inc., Eggenstein, Germany) every fifth passage.

HepG2 cells were transfected with a pcDNA3.1 vector (Invitrogen, SanDiego, CA) containing the coding sequence for Bcl-xL (pcDNA3.1/Bcl-xL) or with a control, neomycin-resistant expression vector pcDNA3.1 by Lipofectin reagent (Invitrogen) according to the manufacturer's instructions. Transgene expression was assessed by Western blot.

2.4. Confirmation of apoptosis

Several methods were used to confirm apoptotic cell death. *In situ* TUNEL assay identified internucleosomal DNA strand breaks characteristic of apoptosis. A TdT-FragEL DNA fragmentation detection kit (Oncogene Research Products, San Diego, CA) was used to detect apoptosis, according to instructions provided by the manufacturer. Cells were harvested by trypsinization and washed once in TBS at indicated times post infection with Ad-TIP30 with mock as control. Then cells were fixed by 4% formaldehyde/PBS at a cell density of 1×10^6. Proteinase K was added (20 μg/ml), incubating at room temperature for no more than 5 min. Cells were in turn equilibrated by 1 × TdT equilibration buffer for 10–30 min. At this end, cells were incubated in TdT reaction mixture at 37 °C, 5% CO_2 for 1–1.5 h. Afterward, cells were analyzed on a flow cytometry equipped with a 488 nm argon ion laser source (Trevigen, Inc. Gaithersburg, MD).

2.5. Measurement of mitochondrial transmembrane potential (Δψm)

The detection of mitochondrial membrane potential (Δψm) was determined according to the instruction of Trevigen (Trevigen, Inc. MD.). Cells were stained with the fluorochrome 5,5′, 6-6′tetrachloro-1, 1′, 3, 3′-tetraethylbenzimidazolcarbo-cyanine iodide (JC-1 (1.25 μg/ml)). HepG2 cells incubated in six-well plates were washed with PBS (phosphate-buffered saline), then 1 ml reaction buffer/well mixed by 1 μl DePsipher™ (Trevigen) was incubated at 37 °C, 5% CO_2 for 15–20 min. Finally, cells were observed immediately under confocal laser scanning microscopy using a fluorescent long-pass filter. In healthy cells, the mitochondria appeared red following aggregation of the DePsipher within the mitochondria. The red aggregates had a maximal emission at 590 nm (FL-2). In dying cells or cells with disrupted potential, the dye remained in its monomeric form in the cytoplasm and would appear green with a maximal emission at 530 nm (FL-1). Alternately, disrupted membrane potential was also assayed by flow cytometry (Trevigen).

2.6. Isolation of cytosol and mitochondrial fractions

Cells were washed twice with PBS, and harvested by centrifugation (500 ×*g* for 5 min). Cell pellets were resuspended in 1000 μl of cytosol extraction buffer (Biovision, Mountain View, CA). Cell homogenates were prepared by disrupting cells in a Dounce glass homogenizer (30–50 strokes) on ice. Unlysed cells and nuclei were subjected at 700 ×*g* for 10 min at 4 °C. The supernatant, which contained mitochondria, was collected and subjected to further centrifugation at 10,000 ×*g* for 30 min. The pellet and the supernatant represented mitochondrial and cytosolic fractions, respectively.

2.7. Western blot analysis and immunoprecipitation

Briefly, the protein content of cell extracts was determined by the Bradford assay [30] (Bio-Rad, Sydney, New SouthWales, Australia). Equal amount of protein loading was further controlled by Coomassie Blue staining of gels. A total of 20–50 μg of protein was electrophoresed on 10–15% SDS-PAGE gels and transferred to polyvinylidene difluoride membranes (Immobilon-P; Millipore, Eschborn, Germany). Membranes were blocked with 5% fat-free milk powder in TBST containing 0.05% Tween 20 (for 1 h) and incubated with specific antibodies against caspase-3, caspase-8, caspase-9, XIAP, poly (ADP-ribose) polymerase, cytochrome *c*, AIF, and Smac/DIABLO overnight at 4 °C. The probed blots were washed and incubated with a horseradish peroxidase-coupled anti-rabbit or anti-mouse IgG, and then visualized by ECL Advance Western Blotting Detection Kit (GE Healthcare, UK).

For immunoprecipitation, cells were lysed as described previously (Biovision, Mountain View, CA). Lysates were cleared by centrifugation at 14,000 ×*g* for 10 min at 4 °C and protein concentration was determined. Cytosol cell lysates were incubated with anti-Smac antibody or anti-caspase-3 antibody (2.5 μl) and protein A Sepharose (GE Healthcare, UK) overnight at 4 °C. The beads were washed three times with 500 μl of lysis buffer and resuspended in 25 μl of a 3× sample buffer containing 1.5% β-mercaptoethanol. After addition of 25 μl of 1× sample buffer, beads were boiled for 5 min at 95 °C and then pelleted by short spin. 50 μl of the supernatant were used for SDS-PAGE.

2.8. RNA interference

The sequences against human Bax were initially synthesized according to human Bax cDNA sequence using the Silencer™ kit (Ambion, Austin, TX). The Bax DNA target sequence for *si*RNA design was 5′-AACTGATCAGAACCATCATGG-3′. Nonspecific control *si*RNA (target 5′GCATTGTATGCGATCGCAGAC') was acquired. The transfection of siRNA oligonucleotides was performed with Lipofectamine 2000 (Invitrogen) according to the manufacturer's recommendations. Forty-eight hours after transfection, the cells were treated with Ad-TIP30. At the end of treatment, the cells were harvested for experiments.

3. Results

3.1. TIP30-induced inner mitochondrial membrane permeabilization and release of Smac/DIABLO, cytochrome c and AIF

Hallmarks of the mitochondrial apoptosis pathway (intrinsic pathway) are the release of cytochrome *c* from the mitochondrial intermembrane space into the cytosol and the dissipation of the electrochemical gradient on the inner mitochondrial membrane (inner mitochondrial membrane permeabilization) [33]. Fig. 1A and B showed that TIP30 (20 MOI) induced outer mitochondrial membrane permeabilization in HepG2 cells as measured by flow cytometry and observed by confocal laser scanning microscopy using the fluorochrome JC-1. The first significant dissipation of Δψm was evident 2 h after stimulation. Moreover, TIP30 (20 MOI, 0–24 h) triggered the release of cytochrome *c* into the cytosol (Fig. 1C). Besides cytochrome *c*, AIF and Smac/DIABLO were presented within the mitochondria and released after apoptotic stimuli (Fig. 1D and E). TIP30 triggered an early (2 h) release of Smac/DIABLO that was not abrogated by the pan-caspase inhibitor z-VAD-fmk. This indicated that TIP30-induced Smac/DIABLO release was an early event that occurred before and independent of caspase activation (Fig. 1E).

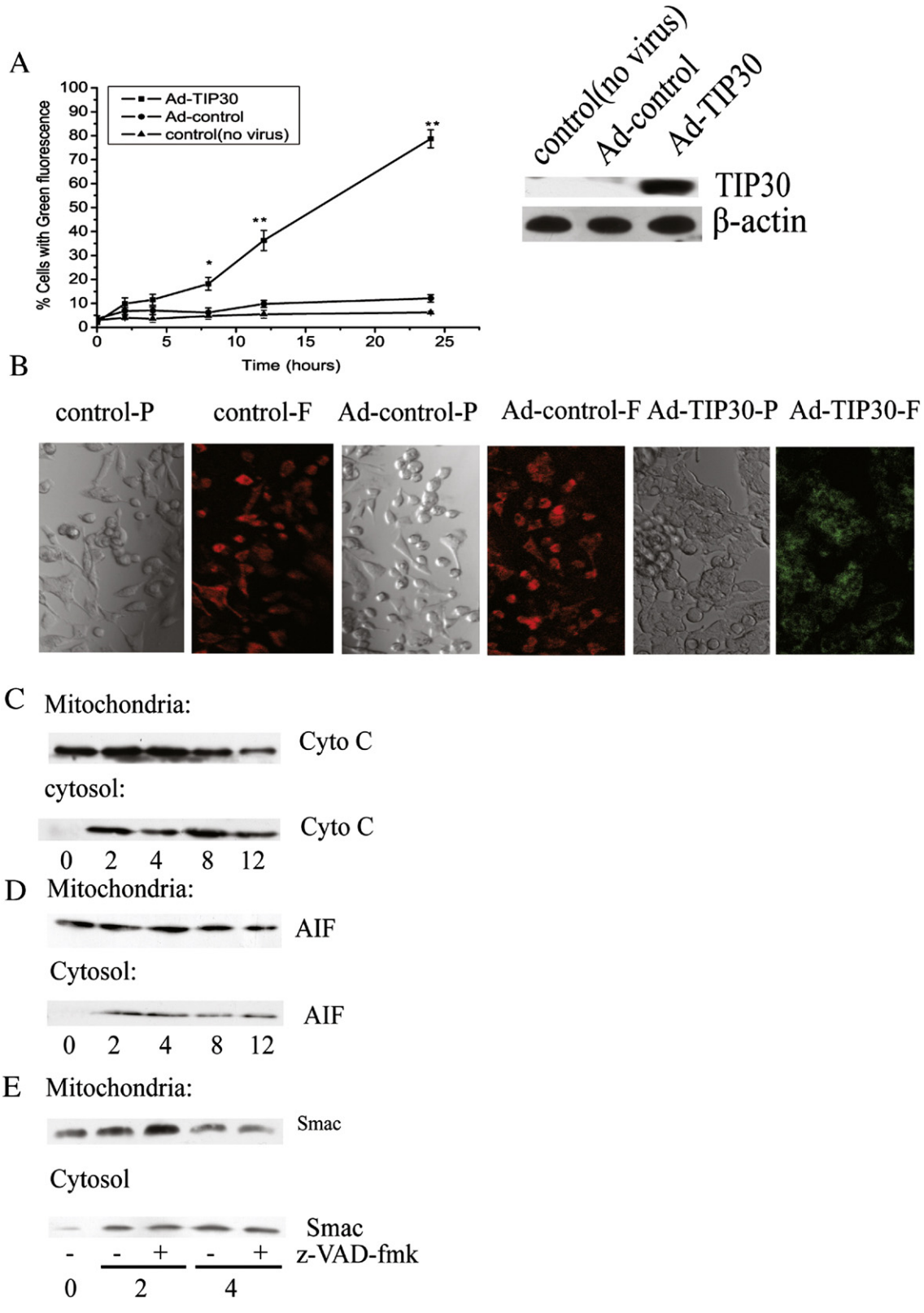
3.2. TIP30-induced apoptosis was dependent on the activation of caspases

To elucidate whether caspase activation was required for TIP30-induced apoptosis, cells were pre-incubated with the broad-spectrum caspase inhibitor benzyloxycarbonyl-Val-Ala-Asp (OMe)-fluoromethyl ketone (z-VAD-fmk). Caspase inhibition led to a complete inhibition of TIP30-induced DNA fragmentation (Fig. 2A), proving the evidence of caspases in this TIP30-mediated apoptosis. To explore whether TIP30-triggered apoptosis followed the extrinsic pathway including activation of the initiator caspase-8 or the intrinsic pathway under involvement of mitochondria and the initiator caspase-9, we examined the activation of these caspases by Western blot assay. The caspase-8 cleavage did not appear until 24 h after stimulation (Fig. 2B). In contrast, Fig. 2C illustrated the time-dependent activation of caspase-9 by TIP30 (20 MOI). The processing of procaspase-9 to the active forms (p37) occurred after only 4 h. To test whether caspase-3, an important effector caspase, was activated downstream of caspase-9, the processing of caspase-3 and poly (ADP-ribose) polymerase cleavage in response to

TIP30 was demonstrated. As shown in Fig. 2C, Ad-TIP30 treatment caused proteolytic cleavage of both caspase-3 and PARP in a time-dependent manner. On the other hand, inhibition of caspase-9 by the caspase-9 inhibitor z-LEHD-fluoromethyl ketone (z-LEHD-fmk) (50 μM) led to an inhibition of caspase-3 activity induced by Ad-TIP30 (Fig. 2C).

3.3. Membrane translocation of Bax was essential for TIP30-mediated apoptosis and loss of Bax inhibited mitochondrial release of Smac/DIABLO and cytochrome c

In normal cells, the Bax protein exists as an inactive form in the cytosol, but it can be induced to change conformation and



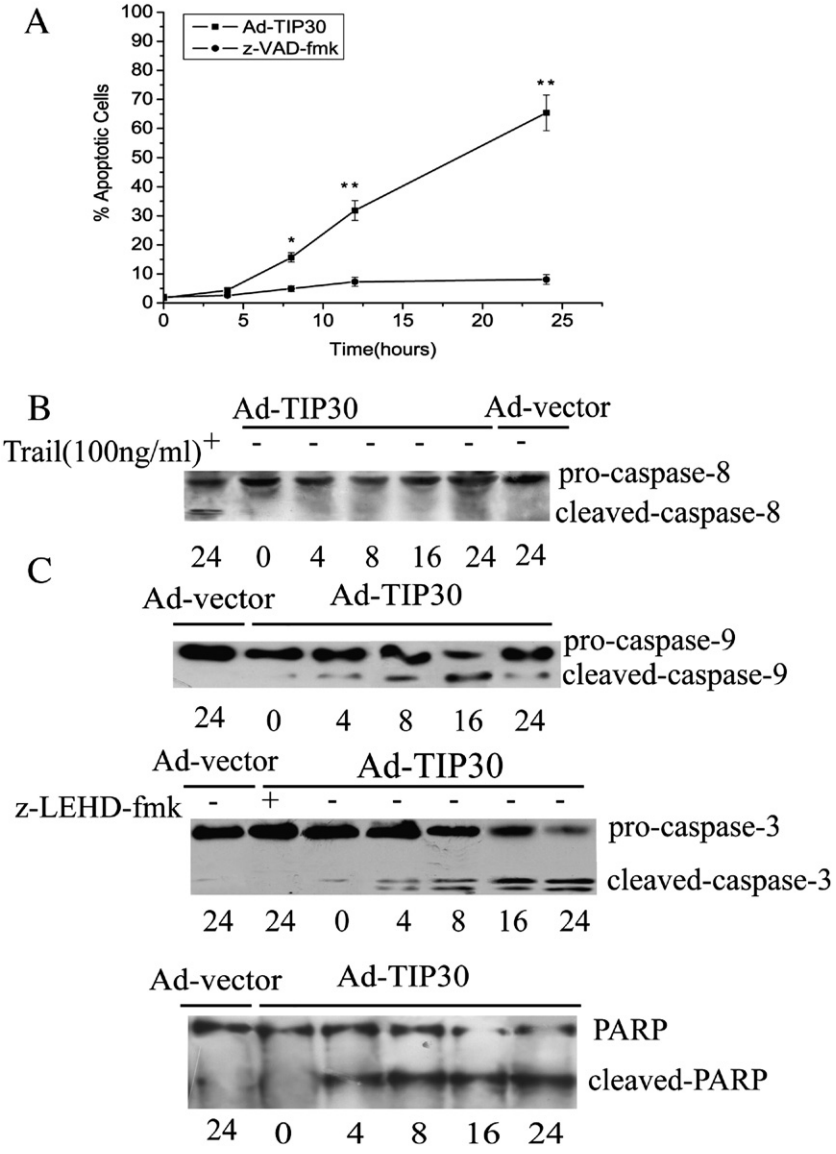


Fig. 2. TIP30-induced apoptosis is dependent on the activation of caspases. A, Inhibition of Ad-TIP30-induced apoptosis by the caspase inhibitor z-VAD-fmk. Cells were infected with Ad-TIP30 (50 MOI, 0–24 h), or pretreated with z-VAD-fmk (25 μM, 1 h) and then stimulated by Ad-TIP30. Apoptotic cells were quantified by flow cytometry. B and C, Representative Western blots showed time-dependent (0–24 h) cleavage as follows: B, Procaspase-8 isoforms were not cleaved to the p42 and p44 cleavage products in cells treated with Ad-TIP30 and Ad-vector. Positive control: HCC cells were treated with 100 ng/ml of TRAIL for 24 h. C, Procaspase-9 was cleaved to the active cleaved forms p37. Procaspase-3 was cleaved to p20/17 and 116-kDa PARP was cleaved to its p89 cleavage product after treatment with Ad-TIP30 (20 MOI). Moreover, cells were treated by a caspase-9 inhibitor (Casp 9IH) in addition to Ad-TIP30 (20 MOI) for 24 h. Whole cell extracts were analyzed by Western blots. Cells were lysed and proteins were separated by SDS-PAGE as described in "Materials and methods". Data represented means and S.E. from three independent experiments. $P<0.05$(*) and $P<0.01$(**) indicated a statistically significant difference with various treatment.

translocate to the mitochondria in response to certain apoptotic stimuli [34]. We took advantage of a Bax-interfered cell line (Bax *si*RNA cells) derived from HepG2/Bax$^{+/+}$ cells. Reduction of Bax expression in HepG2/Bax*si* cells was first confirmed by Western blot assay (Fig. 3A). Forty-eight hours after transfection, HepG2 cells were treated with Ad-TIP30. Most HepG2/

Fig. 1. Ad-TIP30 induces inner mitochondrial membrane permeabilization and the mitochondrial release of Smac/DIABLO, cytochrome *c* and AIF. A, Left lane: Ad-TIP30 led to a time-dependent dissipation of $\Delta\psi m$. HepG2 cells were incubated with Ad-TIP30 (20 MOI) for the indicated time periods and subsequently loaded with the fluorochrome JC-1 (0.25 μg/ml). Cells showing predominantly green fluorescence were quantified by FACS analysis. Right lane: the expression of TIP30 in HepG2 cells treated with Ad-TIP30. B, Cells were either left untreated (no virus) or infected with Ad-control or Ad-TIP30 (20 MOI) for 24 h and prepared for confocal laser scanning microscopy. Representative pictures are shown. Control cells and cells treated by Ad-control or Ad-TIP30 were viewed by DIC (control-P, Ad-control-P and Ad-TIP30-P) and fluorescence (control-F, Ad-control-F and Ad-TIP30-F). (magnification ×200) C, D and E, Ad-TIP30 led to release of cytochrome *c* (C), AIF (D) or Smac/DIABLO (E) into the cytosol. Cells were treated with Ad-TIP30 (20 MOI) for the indicated times. Cytoplasmic and mitochondrial protein were prepared as described in "Materials and methods" and detected by Western blot analysis. E, cells were pretreated with z-VAD-fmk (25 μM, 1 h) where indicated and subsequently stimulated with Ad-TIP30 (20 MOI) for 2 and 4 h. Cytoplasmic and mitochondrial protein were prepared and analyzed as described in C and D. Representative Western blots are shown. Data represented means and S.E. from three independent experiments. $P<0.05$(*) and $P<0.01$(**) indicated a statistically significant difference with Ad-TIP30 and the control.

control*si* cells underwent apoptosis after 20 h of treatment with Ad-TIP30, whereas little apoptosis was observed in HepG2/Bax*si* cells (Fig. 3A). Treatment of HepG2/Bax*si* cells with Ad-TIP30 for 48 h did not result in significant cell death (data not shown), suggesting that Bax was required for Ad-TIP30-induced apoptosis in HepG2 cells. Moreover, the significant dissipation of $\Delta\psi m$ was abrogated in HepG2/Bax*si* cells (Fig. 3B).

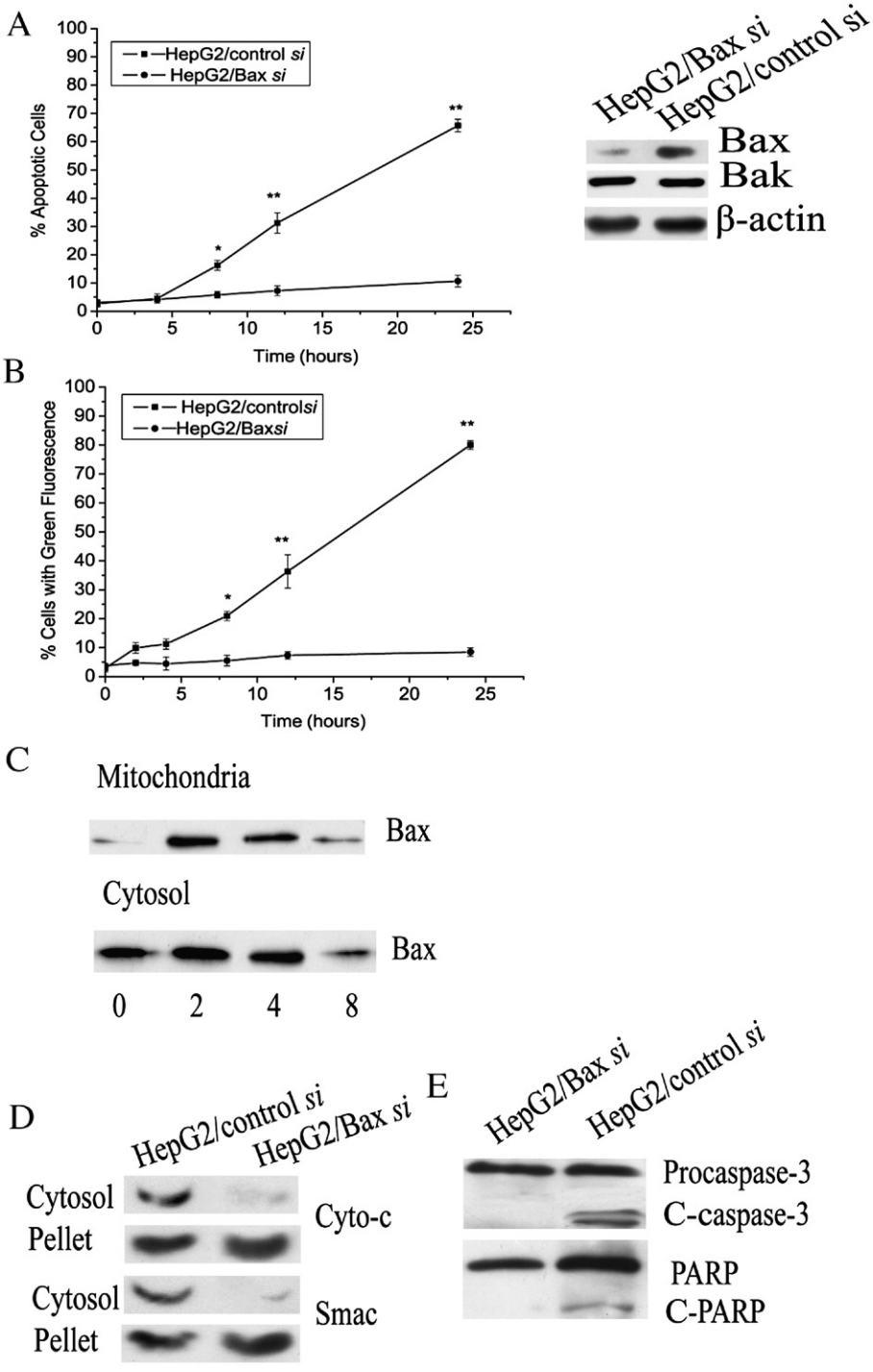


Fig. 3. Requirement of Bax translocation in TIP30-induced apoptosis. A, Left lane: Suppression of Ad-TIP30-induced apoptosis in HepG2/Bax*si* cells. HepG2 cells were transfected with Bax *si*RNA (Bax*si*) or control *si*RNA (control*si*). After 48 h, cells were treated with Ad-TIP30 (50 MOI) for indicated time periods, and apoptotic cells were quantified by flow cytometry. Right lane: expression of Bax and Bak in HepG2/Bax*si* and HepG2/control*si* cells was detected by Western analysis. B, Time-dependent dissipation of $\Delta\psi m$ was quantified by FACS analysis as previous description. C, Change of Bax distribution after Ad-TIP30 treatment. HepG2 cells were treated by Ad-TIP30 (20 MOI), and Bax protein was detected on a Western blot from cytosol and mitochondrial extracts. D, Subcellular fraction was performed on cells after Ad-TIP30 treatment for 4 h in HepG2/Bax*si* and HepG2/control*si* cells. Pellets and supernatants (cytosol fraction) were analyzed by Western blot for Smac/DIABLO and cytochrome *c*. E, Western blotting was performed to analyze for the cleavage of procaspase-3 and PARP. Data represented means and S.E. from three independent experiments. $P<0.05$(*) and $P<0.01$(**) indicated a statistically significant difference with various treatment.

To explore whether Bax translocation was involved in TIP30-mediated apoptosis, HepG2/Bax$^{+/+}$ cells were treated with Ad-TIP30, and Bax localization was examined by subcellular fractionization followed by Western blot. The Bax protein was located in the cytosol before Ad-TIP30 treatment and was redistributed to mitochondria after Ad-TIP30 treatment (Fig. 3C), suggesting that Ad-TIP30 induced Bax translocation. These results suggest that Bax translocation from the cytosol to mitochondria was required for Ad-TIP30-induced apoptosis.

One of the events mediated by Bax is the release of cytochrome *c* from mitochondria, followed by procaspase-9 activation. The Smac/DIABLO protein is also redistributed from mitochondria to cytosol during mitochondria-initiated apoptosis, concurrent with cytochrome *c* relocalization [35]. Therefore, we investigated whether reduction of the Bax could block Smac/DIABLO and cytochrome *c* release and procaspase-9 or procaspase-3 activation in the TIP30-signaling pathway. As shown in Fig. 3D, Bax down-regulation almost completely inhibited the apoptotic stimuli-induced cytochrome *c* and Smac release. Moreover, the cleaved caspase-3 and PARP appeared in HepG2/control*si* cells 4 h after Ad-TIP30 treatment. In contrast, procaspase-3 cleavage was absent in HepG2/Bax*si* cells (Fig. 3E). Consistent with the finding that cytochrome *c* release was absent in these cells, the data showed that procaspase-3 and

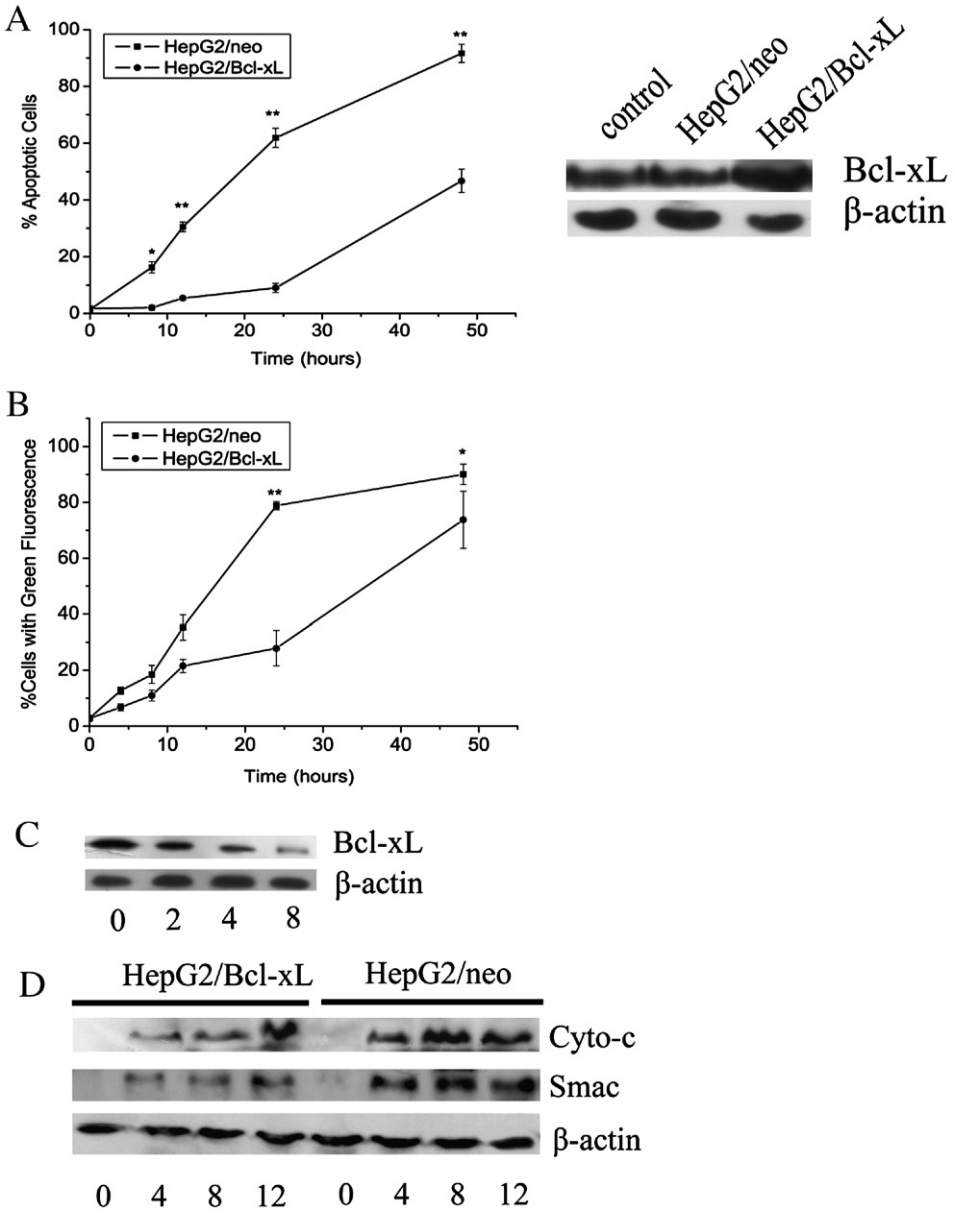


Fig. 4. Bcl-xL delays mitochondrial Smac/DIABLO release and onset of apoptosis as well as dissipation of Δψm. A, Left lane: control cells (HepG2/neo) and cells overexpressing Bcl-xL (HepG2/Bcl-xL) were treated with Ad-TIP30 (50 MOI). Apoptotic cells were quantified by flow cytometry for the indicated times. Right lane: the level of Bcl-xL in HepG2 cells, cells transfected with a pcDNA3.1 vector or with pcDNA3.1/Bcl-xL. B, Time-dependent dissipation of Δψm was quantified by FACS analysis as previous description. C, Down-regulation of Bcl-xL was detected in cells treated by TIP30 (20 MOI) for the indicated times. D, Cytoplastic proteins, such as cytochrome *c* and Smac/DIABLO, were detected using Western blot analysis. Data represented means and S.E. from three independent experiments. $P<0.05$(*) and $P<0.01$(**) indicated a statistically significant difference with various treatment.

PARP activation in response to Ad-TIP30 were inhibited by the reduction of Bax. These results showed that the mitochondrial pathway was activated by Ad-TIP30 treatment in a Bax-dependent manner.

3.4. Bcl-xL down-regulation was required for TIP30-induced apoptosis

Bcl-xL appears to inhibit cell death by blocking the formation of these cytochrome *c*-releasing pores. Previous studies had shown that Bcl-xL might be down-regulated during apoptosis induced by chemotherapy reagents [36,37]. After treatment with Ad-TIP30, the level of Bcl-xL was markedly decreased in HepG2/neo cells (Fig. 4C). In the apoptotic assay, compared with the control HepG2/neo cells, the HepG2/Bcl-xL cell expressed approximately 2–3-fold higher levels of Bcl-xL (Fig. 4A). Ad-TIP30 treatment induced apoptosis in 60% of control HepG2/neo cells, but little cell death was observed after exposing HepG2/Bcl-xL cells to Ad-TIP30 treatment for 24 h (Fig. 4A). Similarly, dissipation of $\Delta\psi m$ was also affected by overexpression of Bcl-xL (Fig. 4B). Fig. 4D showed that release of cytochrome *c* and Smac/DIABLO was significantly delayed in HepG2/Bcl-xL cells compared with HepG2/neo cells. These results confirmed that Ad-TIP30-induced apoptosis was initiated by mitochondrial release of apoptogenic molecules and regulated principally by Bax and Bcl-xL in HCC cells.

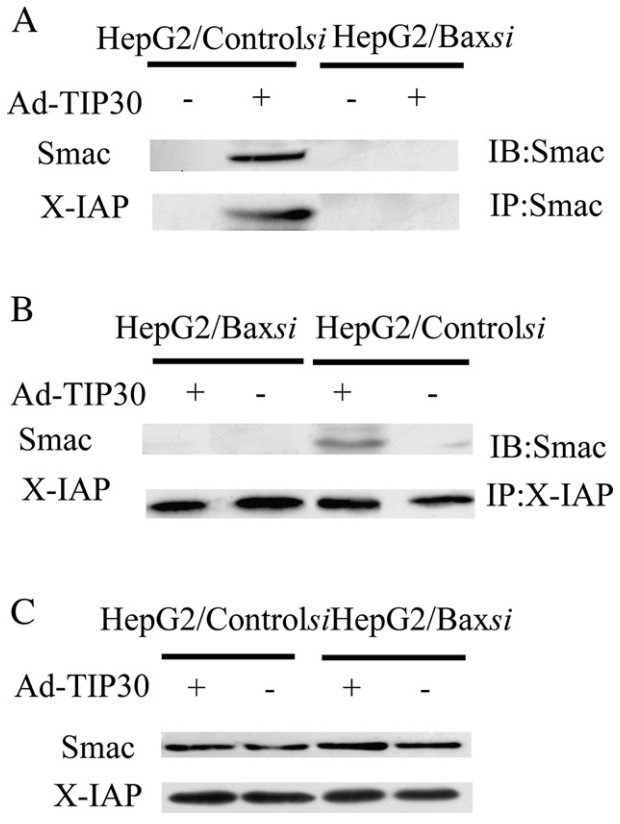


Fig. 5. Interaction between XIAP and Smac/DIABLO in Ad-TIP30-induced apoptosis. A and B, Cells treated with Ad-TIP30 (20MOI) for 4 h and whole cell extracts were examined for Smac/DIABLO and XIAP protein expression on Western blots. Cytosol extracts were immunoprecipitated with anti-XIAP and Smac/DIABLO antibodies, and blotted for Smac/DIABLO and XIAP, respectively. C, Cells treated with Ad-TIP30 for 4 h and whole cell extracts were examined for Smac/DIABLO and XIAP protein expression with Western blots.

3.5. Interaction between XIAP and Smac/DIABLO in Ad-TIP30-induced apoptosis

One of the factors released from mitochondria during apoptosis is the Smac/DIABLO protein, which binds and neutralizes the inhibitory activity of IAPs, especially XIAP, and physically facilitate caspases activation in cancer cells [38,39]. Immunoblotting revealed that Smac/DIABLO and XIAP were readily detectable in whole cell extracts (Fig. 5C). Association between Smac/DIABLO and XIAP was analyzed by coimmunoprecipitation of cytosol extracts obtained from HCC cells before and after Ad-TIP30 treatment. More importantly, immunoprecipitation of XIAP coimmunoprecipitated cytosolic Smac/DIABLO in HepG2/control*si* cells treated with Ad-TIP30 (Fig. 5). These results supported the hypothesis that Bax-dependent release of Smac/DIABLO from mitochondria enabled Smac/DIABLO to interact with XIAP thereby disrupting the XIAP–caspase interaction, thus allowing caspase autocleavage and functional activation.

4. Discussion

To investigate the signaling pathway of TIP30-mediated apoptosis, we took advantage of a recombinant adenovirus-mediated delivery system. Adenovirus was used to deliver genes into mammalian cells, particularly where there was a requirement for high-level expression of transgene products in cultured cells. Full length TIP30 was cloned into a replication deficient recombinant adenovirus and the expression of TIP30 was verified by Western blot analysis. A higher expression level of TIP30 was observed in cells transduced with Ad-TIP30 as compared to negative control (Fig. 1A).

We had shown that TIP30 underwent P53-dependent cell death cascade [11,12], consistent with the idea that TIP30-induced apoptosis was mediated by a common mitochondrial pathway. Most importantly, we had shown that mitochondrial cell death events were activated by TIP30 stimulation in a Bax-dependent manner and that apoptosis was ignored in HepG2/Bax*si* cells. This is the first report showing that TIP30 triggered an intrinsic apoptosis in HCC cells that used mitochondria as a signaling integrator and Smac/DIABLO as a main TIP30-signaling molecule. Based on the results from previous reports by others and our present studies, we proposed a sequence of signal transduction events involved in TIP30 (Fig. 6).

The cellular signal generated by TIP30 used mitochondria to culminate in the common execution pathway triggered by cytochrome *c*. The cytosolic cytochrome *c* formed a complex with Apaf-1 and procaspase-9 in the presence of ATP, which led to the activation of caspase-9. Activated caspase-9 further activated other caspases such as caspase-3, and then resulted in apoptosis. We first studied the mitochondrial membrane potential ($\Delta\psi m$) upon Ad-TIP30 exposure. Thus, it appeared that the mitochondria were an important target for TIP30-induced apoptosis (Fig. 1).

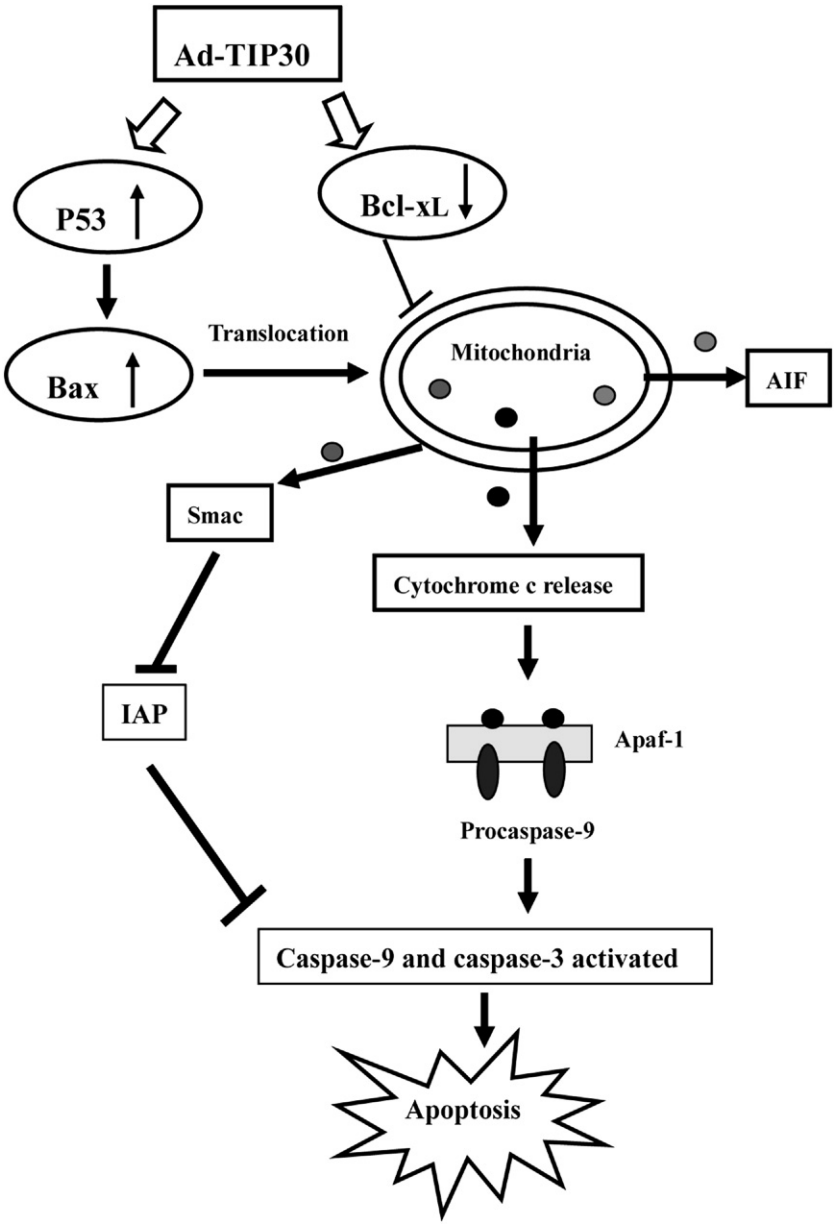


Fig. 6. Proposed model of Ad-TIP30-induced Bax translocation in regulating apoptosis. Stimulation of P53 resulted in up-regulation of Bax. Then Bax translocated from the cytoplasm to the outer mitochondrial membrane, where it stimulated release of both cytochrome *c*, AIF and Smac/DIABLO. Because Smac efficiently removed XIAP from active caspases, and in turn promoted their activity. Procaspase-9 subsequently underwent transcatalytic processing, resulting in active caspase-9 that cleaved its substrates, procaspase-3. Initial processing of procaspase-3 produced an active caspase-3 intermediate, whereas Bcl-xL could modulate cell death by inhibiting release of cytochrome *c* and Smac.

Bcl-2 family members are major regulators of the mitochondria-initiated caspase activation pathway [40]. The present study was designed to gain insights into the role of these Bcl-2 family members in TIP30-mediated apoptosis. Whitman [8] had previously reported that TIP30/CC3 induced apoptosis in a Bcl-2-inhibitable fashion involving perturbation of mitochondria in SCLC cells. Previous studies showed that induction of apoptosis by diverse antitumor drugs in different cellular systems was associated with the induction of Bax translocation. In normal cells, the Bax existed in an inactive form mainly in the cytosol but could be induced to change conformation and translocate into the mitochondria in response to certain apoptotic stimuli. The conformationally changed Bax protein oligomerized on the outer mitochondrial membrane and induced the release of apoptogenic molecules into the cytoplasm [41,42]. To determine whether Bax translocation was involved in TIP30-induced apoptosis, we performed subcellular fractionation and Western blot analysis on HepG2 cells infected by Ad-TIP30 at several time intervals. This conclusion was based on the following observations: (a) Ad-TIP30 treatment causes a time-dependent translocation of Bax in wild-type cells (Fig. 3C), (b) the HepG2/Bax*si* cells prevented TIP30-induced HCC cell death when compared with HepG2/control*si* cells (Fig. 3A), and (c) data indicated that membrane translocation of Bax led to

activation of caspase-3 and PARP (Fig. 3E). These data suggested that translocation of Bax was necessary and sufficient for complete processing mitochondrial cascade in the TIP30-mediated cell death pathway.

It was well established in the literature that Bcl-xL was highly expressed in many cell types, especially in HCC cells. It possesses properties of attenuating cell death at the mitochondrial level, preventing the release of cytochrome *c* and the loss of $\Delta\psi m$ [43]. Indeed, resistance to chemotherapy was related to increased levels of the mitochondria-protecting proteins Bcl-2 and Bcl-xL. Previous reports demonstrated that ectopic expression of Bcl-xL in cancer cells conferred resistance to apoptosis against a variety of death-inducing agents [44,45]. Similarly, our data indicated that infected by Ad-TIP30, Bcl-xL protein level decreased in HepG2 cells, implying that overexpression of TIP30 might trigger apoptosis at least by down-regulating Bcl-xL in HCC cells (Fig. 4C). Changes in the $\Delta\psi m$ were retarded by overexpression of Bcl-xL, which resulted in a marked delay in the kinetics of apoptosis (Fig. 4A and B). It would be consistent with induction of changes in Bax by Ad-TIP30 (Fig. 3). In conclusion, overexpression of Bcl-xL was associated with suppression of cytochrome *c*/Smac/DIABLO release (Fig. 4D).

One of the key regulatory steps for apoptosis is the activation of caspase. Active caspase then cleaved many important intracellular substrates, leading to the characteristic morphological changes associated with apoptotic cells [46]. To determine whether mitochondiral/caspase-9 pathway was activated in Ad-TIP30-induced apoptosis in HepG2 cells, we analyzed the cleavage product of caspase-3, -9 and PARP by western blotting. The result showed that in HepG2 cells, both caspases were activated during apoptosis as judged by appearance of cleavage products from procaspase (Fig. 2C). Previous studies *in vitro* had suggested that caspase-9 could directly process procaspase-3 into its active form by two distinct cleavage events [47]. As expected, we had shown that inhibition of caspase-9 blocked caspase-3 processing and activation in HepG2 cells (Fig. 2C). Consistent with earlier study documented the specific role of caspase-3 as an amplifier of mitochondrial cytochrome *c* release and of morphological changes of nuclei and DNA fragmentation during adenovirus-induced apoptosis in hepatocellular carcinoma cells [48].

Smac/DIABLO was identified as a mitochondrial factor involved in apoptosis by removing XIAP inhibition on caspases. During stress-induced apoptosis, Smac/DIABLO was released along with cytochrome *c* from mitochondria into the cytosol. Although released cytochrome *c* contributed to the formation of the apoptosome and thereby to the initiation of the caspase-3-dependent caspase cascade. Smac/DIABLO promoted caspase activity by binding to the XIAP in a manner that displaced caspases from their inhibitor XIAP [49–52]. In this setting, Smac/DIABLO release was sufficient to promote full caspase activation. The function of Smac/DIABLO in the cytosol appeared to dissociate caspase–XIAP interaction, as shown by coimmunoprecipitation of XIAP and Smac/DIABLO (Fig. 5).

Previous studies have demonstrated that chemical substance such as O-Trensox, Doxorubicin, MG132 could facilitate HCC cells to undergo apoptosis by P53 elevation, increase of pro-apoptotic members and down-regulation of anti-apoptotic members of Bcl-2 family, or moreover by reduction in mitochondrial transmembrane potential with the consequence of activation of caspase-3 and degradation of PARP [53,54]. We had shown that P53-deficient-Hep3B and P53-mutational-PLC/RPF/5 cells displayed a relatively low apoptotic rate with Ad-TIP30 infection. Thus, P53 might be a major factor regulated by TIP30 and enhanced the process of apoptosis. Nevertheless, the apoptotic pathway of the two kinds of cells required further experiments. It was still worth that we established a common mechanism by which diverse factor predisposed HCC cells to apoptosis. Based on our results and the results from others, we proposed the following model (Fig. 6): upon activation of P53 by TIP30, P53 in turn activated the downstream factor, mostly like Bax. Thereby triggered the translocation of Bax to mitochondria where it promoted the release of cytochrome *c*, AIF and Smac/DIABLO. Smac efficiently removed XIAP from active caspases and procaspase-9 subsequently underwent transcatalytic processing, resulting in active caspase-9. Then it cleaved its substrates, such as procaspase-3, leading to apoptosis.

Given that TIP30 is a promising potential anticancer agent, understanding the contribution of TIP30 to apoptosis is of significance for the development of its therapy for human hepatoblastoma. Impairment in mitochondrial activation is frequently associated with cancer development, such as mutations in Bax and loss of function of Apaf-1. Therefore, Bax and Smac/DIABLO represent potential therapeutic targets to bypass the involvement of the mitochondrial pathway and improved TIP30 cancer therapy.

Acknowledgments

We thank Dr. Hua Xiao (University of Nebraska Medical Center, Omaha, USA) for kindly providing the plasmid (a pcDNA3 vector with full-length *tip30* gene) and anti-TIP30 antibody. We also thank Dr. Kathleen Cooley and Dr. Xiangyuan He from Oklahoma Medical Research Foundation for critical review of the manuscript.

References

[1] H. Xiao, Y. Tao, A cofator, Tip30, specifically enhances HIV-1 Tat-activated transcription, Proc. Natl. Acad. Sci. 95 (1998) 2146–2151.
[2] E. Shtivelman, A link between metastasis and resistance to apoptosis of variant small cell lung carcinoma, Oncogene 14 (1997) 2167–2173.
[3] R. NicAmhlaoibh, E. Shtivelman, Metastasis suppressor CC3 inhibits angiogenic properties of tumor cells in vitro, Oncogene 20 (2001) 270–275.
[4] J. Zhao, H. Ni, Y. Ma, L. Dong, J. Dai, F. Zhao, X. Yan, B. Lu, H. Xu, Y. Guo, TIP30/CC3 expression in breast carcinoma: relation to metastasis, clinicopathologic parameters, and P53 expression, Hum. Pathol. 38 (2007) 293–298.
[5] M. Ito, C. Jiang, K. Krumm, X. Zhang, J. Pecha, J. Zhao, Y. Guo, R.G. Roeder, H. Xiao, TIP30 deficiency increases susceptibility to tumorigenesis, Cancer Res. 63 (2003) 8763–8767.
[6] J. Pecha, D. Ankrapp, C. Jiang, W. Tang, I. Hoshino, K. Bruck, K.U. Wagner, H. Xiao, Deletion of Tip30 leads to rapid immortalization of murine mammary epithelial cells and ductal hyperplasia in the mammary gland, Oncogene 28 (2007) (Electronic publication).
[7] C. Jiang, M. Ito, V. Piening, K. Bruck, R.G. Roeder, H. Xiao, TIP30 interacts with an estrogen receptor alpha-interacting coactivator CIA and regulates c-myc transcription, J. Biol. Chem. 279 (2004) 22789–27781.

[8] S. Whitman, X. Wang, R. Shalaby, E. Shtivelman, Alternatively spliced products CC3 and TC3 have opposing effects on apoptosis, Mol. Cell Biol. 20 (2000) 583–593.
[9] H. Xiao, V. Palhan, Y. Yang, R.G. Roeder, TIP30 has an intrinsic kinase activity required for up-regulation of a subset of apoptotic genes, EMBO J. 19 (2000) 956–963.
[10] J. Zhao, X. Zhang, M. Shi, H. Xu, J. Jin, H. Ni, S. Yang, J. Dai, M. Wu, Y. Guo, TIP30 inhibits growth of HCC cell lines and inhibits HCC xenografts in mice in combination with 5-FU, Hepatology 44 (2006) 205–215.
[11] C. Jiang, J. Pecha, I. Hoshino, D. Ankrapp, H. Xiao, TIP30 mutant derived from hepatocellular carcinoma specimens promotes growth of HepG2 cells through up-regulation of N-cadherin, Cancer Res. 67 (2007) 3574–3582.
[12] M. Shi, X. Zhang, P. Wang, H.W. Zhang, B.H. Zhang, M.C. Wu, TIP30 regulates apoptosis-related genes in its apoptotic signal transduction pathway, World J. Gastroenterol. 11 (2005) 221–227.
[13] D.E. Fisher, The p53 tumor suppressor: critical regulator of life and death in cancer, Apoptosis 6 (2001) 7–15.
[14] M. Schuler, E. Bossy-Wetzel, J.C. Goldstein, P. Fitzgerald, D.R. Green, p53 induces apoptosis by caspase activation through mitochondrial cytochrome *c* release, J. Biol. Chem. 275 (2000) 7337–7342.
[15] A.K. Ghosh, M. Majumder, R. Steele, T.J. Liu, R.B. Ray, MBP-1 mediated apoptosis involves cytochrome *c* release from mitochondria. Oncogene 21 (2002) 2775–2784.
[16] H. Dussmann, D. Kogel, M. Rehm, J.H. Prehn, Mitochondrial membrane permeabilization and superoxide production during apoptosis: a single-cell analysis. J. Biol. Chem. 278 (2003) 12645–12649.
[17] P. Delivani, S.J. Martin, Mitochondrial membrane remodeling in apoptosis: an inside story, Cell Death Differ. 13 (2006) 2007–2010.
[18] J.C. Wilkinson, E. Cepero, L.H. Boise, C.S. Duckett, Upstream regulatory role for XIAP in receptor-mediated apoptosis, Mol. Cell Biol. 24 (2004) 7003–7014.
[19] Y. Morizane, R. Honda, K. Fukami, H. Yasuda, X-linked inhibitor of apoptosis functions as ubiquitin ligase toward mature caspase-9 and cytosolic Smac/DIABLO, J. Biochem. 137 (2005) 125–132.
[20] K. Kandasamy, S.M. Srinivasula, E.S. Alnemri, C.B. Thompson, S.J. Korsmeyer, J.L. Bryant, R.K. Srivastava, Involvement of proapoptotic molecules Bax and Bak in tumor necrosis factor-related apoptosis-inducing ligand (TRAIL)-induced mitochondrial disruption and apoptosis: differential regulation of cytochrome *c* and Smac/DIABLO release, Cancer Res. 63 (2003) 1712–1721.
[21] Y. Deng, Y. Lin, X. Wu, TRAIL-induced apoptosis requires Bax-dependent mitochondrial release of Smac/DIABLO, Genes Dev. 16 (2002) 33–45.
[22] R.T. Uren, G. Dewson, C. Bonzon, T. Lithgow, D.D. Newmeyer, R.M. Kluck, Mitochondrial release of pro-apoptotic proteins: electrostatic interactions can hold cytochrome *c* but not Smac/DIABLO to mitochondrial membranes, J. Biol. Chem. 280 (2005) 2266–2274.
[23] H.L. Vieira, P. Boya, I. Cohen, C. El Hamel, D. Haouzi, S. Druillenec, A.S. Belzacq, C. Brenner, B. Roques, G. Kroemer, Cell permeable BH3-peptides overcome the cytoprotective effect of Bcl-2 and Bcl-X(L), Oncogene 21 (2002) 1963–1977.
[24] Y. Tsujimoto, S. Shimizu, Bcl-2 family: life-or-death switch, FEBS 466 (2000) 6–10.
[25] D.S. McClintock, M.T. Santore, V.Y. Lee, J. Brunelle, G.R. Budinger, W.X. Zong, C.B. Thompson, N. Hay, N.S. Chandel, Bcl-2 family members and functional electron transport chain regulate oxygen deprivation-induced cell death, Mol. Cell Biol. 22 (2002) 94–104.
[26] B.J. Kim, S.W. Ryu, B.J. Song, JNK- and p38 kinase-mediated phosphorylation of Bax leads to its activation and mitochondrial translocation and to apoptosis of human hepatoma HepG2 cells, J. Biol. Chem. 281 (2006) 21256–21265.
[27] T.A. Precht, R.A. Phelps, D.A. Linseman, B.D. Butts, S.S. Le, T.A. Laessig, R.J. Bouchard, K.A. Heidenreich, The permeability transition pore triggers Bax translocation to mitochondria during neuronal apoptosis, Cell Death Differ. 12 (2005) 255–265.
[28] I. Lebedeva, R. Rando, J. Ojwang, P. Cossum, C.A. Stein, Bcl-xL in prostate cancer cells: effects of overexpression and down-regulation on chemosensitivity, Cancer Res. 60 (2000) 6052–6060.
[29] A.M. Petros, E.T. Olejniczak, S.W. Fesik, Structural biology of the Bcl-2 family of proteins, Biochimica et Biophysica Acta (BBA)–Mol. Cell Res. 1644 (2004) 83–94.
[30] M.M. Bradford, A rapid and sensitive method for the quantitation of microgram quantities of protein utilizing the principle of protein-dye binding, Anal. Biochem. 72 (1976) 248–254.
[31] C. Craig, M. Kim, E. Ohri, R. Wersto, D. Katayose, Z. Li, Y.H. Choi, B. Mudahar, S. Srivastava, P. Seth, K. Cowan, Effects of adenovirus-mediated p16INK4A expression on cell cycle arrest are determined by endogenous p16 and Rb status in human cancer cells, Oncogene 16 (1998) 265–272.
[32] C. Craig, R. Wersto, M. Kim, E. Ohri, Z. Li, D. Katayose, S.J. Lee, J. Trepel, K. Cowan, P. Seth, A recombinant adenovirus expressing p27Kip1 induces cell cycle arrest and loss of cyclin-Cdk activity in human breast cancer cells, Oncogene 14 (1997) 2283–2289.
[33] D. Arnoult, P. Parone, J.C. Martinou, B. Antonsson, J. Estaquier, J.C. Ameisen, Mitochondrial release of apoptosis-inducing factor occurs downstream of cytochrome *c* release in response to several proapoptotic stimuli, J. Cell Biol. 159 (2002) 923–929.
[34] S.S. Smaili, Y.T. Hsu, K.M. Sanders, J.T. Russell, R.J. Youle, Bax translocation to mitochondria subsequent to a rapid loss of mitochondrial membrane potential, Cell Death Differ. 8 (2001) 909–920.
[35] Y. Deng, X. Wu, Peg3/Pw1 promotes p53-mediated apoptosis by inducing Bax translocation from cytosol to mitochondria, Proc. Natl. Acad. Sci. U. S. A. 97 (Oct 2000) 12050–12055.
[36] I. Mohiuddin, X. Cao, B. Fang, M. Nishizaki, W.R. Smythe, Significant augmentation of pro-apoptotic gene therapy by pharmacologic bcl-xl down-regulation in mesothelioma, Cancer Gene Ther. 8 (2001) 547–554.
[37] C.E. Jenkins, A. Swiatoniowski, M.R. Power, T.J. Lin, *Pseudomonas aeruginosa*-induced human mast cell apoptosis is associated with up-regulation of endogenous Bcl-x_S and down-regulation of Bcl-xl, J. Immunol. 177 (2006) 8000–8007.
[38] V.M. Dirsch, I.M. Muller, S.T. Eichhorst, G.R. Pettit, Y. Kamano, M. Inoue, J.P. Xu, Y. Ichihara, G. Wanner, A.M. Vollmar, Cephalostatin 1 selectively triggers the release of Smac/DIABLO and subsequent apoptosis that is characterized by an increased density of the mitochondrial matrix, Cancer Res. 63 (2003) 8869–8876.
[39] C. Du, M. Fang, Y. Li, L. Li, X. Wang, Smac, a mitochondrial protein that promotes cytochrome *c*-dependent caspase activation by eliminating IAP inhibition, Cell 102 (2000) 33–42.
[40] M. Forte, P. Bernardi, The permeability transition and BCL-2 family proteins in apoptosis: co-conspirators or independent agents? Cell Death Differ. 13 (2006) 1287–1290.
[41] V. Gogvadze, S. Orrenius, B. Zhivotovsky, Multiple pathways of cytochrome *c* release from mitochondria in apoptosis, Biochim. Biophys. Acta. 1757 (2006) 639–647.
[42] D.R. Green, J.C. Reed, Mitochondrial and apoptosis, Science 281 (1998) 1309–1312.
[43] J. Li, F. Shen, D. Wu, L.X. Wei, Y.Z. Wang, L.H. Shi, Y. Zou, M.C. Wu, Expression level of Bcl-XL critically affects sensitivity of hepatocellular carcinoma cells to LIGHT-enhanced and interferon-gamma-induced apoptosis, Oncol. Rep. 17 (2007) 1067–1075.
[44] T. Shimazu, K. Degenhardt, A. Nur-E-Kamal, J. Zhang, T. Yoshida, Y. Zhang, R. Mathew, E. White, M. Inouye, NBK/BIK antagonizes MCL-1 and BCL-XL and activates BAK-mediated apoptosis in response to protein synthesis inhibition, Genes Dev. 21 (2007) 929–941.
[45] T. Ni Chonghaile, C.G. Concannon, E. Szegezdi, A.M. Gorman, A. Samali, Dexamethasone inhibits apoptosis in C6 glioma cells through increased expression of Bcl-XL, Apoptosis 11 (2006) 1247–1255.
[46] E.A. Slee, C. Adrain, S.J. Martin, Serial killers: ordering caspase activation events in apoptosis, Cell Death Differ. 6 (1999) 1067–1074.
[47] M.M. Zeigler, A.I. Doseff, M.F. Galloway, J.M. Opalek, P.T. Nowicki, J.L. Zweier, C.K. Sen, C.B. Marsh, Presentation of nitric oxide regulates monocyte survival through effects on caspase-9 and caspase-3 activation, J. Biol. Chem. 278 (2003) 12894–12902.
[48] T. Ohuchida, K. Okamoto, K. Akahane, A. Higure, H. Todoroki, Y. Abe, M. Kikuchi, S. Ikematsu, T. Muramatsu, H. Itoh, Midkine protects hepatocellular carcinoma cells against TRAIL-mediated apoptosis through down-regulation of caspase-3 activity, Cancer 100 (2004) 2430–2436.

[49] T.M. Hansen, D.J. Smith, P. Nagley, Smac/DIABLO is not released from mitochondria during apoptotic signalling in cells deficient in cytochrome *c*, Cell Death Differ. 13 (2006) 1181–1190.
[50] L.L. Zhou, L.Y. Zhou, K.Q. Luo, D.C. Chang, Smac/DIABLO and cytochrome *c* are released from mitochondria through a similar mechanism during UV-induced apoptosis, Apoptosis 10 (2005) 289–299.
[51] C. Adrain, E.M. Creagh, S.J. Martin, Apoptosis-associated release of Smac/DIABLO from mitochondria requires active caspases and is blocked by Bcl-2, EMBO J. 20 (2001) 6627–6636.
[52] X.M. Sun, S.B. Bratton, M. Butterworth, M. MacFarlane, G.M. Cohen, Bcl-2 and Bcl-xL inhibit CD95-mediated apoptosis by preventing mitochondrial release of Smac/DIABLO and subsequent inactivation of X-linked inhibitor-of-apoptosis protein, J. Biol. Chem. 277 (2002) 11345–11351.
[53] T.K. Lee, T.C. Lau, I.O. Ng, Tracy Ching-Man Lau, Irene Oi-Lin Ng, Doxorubicin-induced apoptosis and chemosensitivity in hepatoma cell lines, Cancer Chemother. Pharmacol. 49 (2002) 78–86.
[54] S. Emanuele, G. Calvaruso, M. Lauricella, M. Giuliano, G. Bellavia, A. D'Anneo, R. Vento, G. Tesoriere, Apoptosis induced in hepatoblastoma HepG2 cells by the proteasome inhibitor MG132 is associated with hydrogen peroxide production, expression of Bcl-xs and activation of caspase 3, Int. J. Oncol. 21 (2002) 857–865.